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# *IN VITRO* GROWTH OF TISSUES SUITABLE TO THE FORMATION OF BONE AND BONE FORMING TISSUE FORMED THEREBY

## Field of the Invention

This invention relates to the formation of a tissue engineered material using *in vitro* cell culture, in a bioreactor system, in the presence of biomaterials suitable for the induction of new bone formation. This invention further relates to the use of specific forms of reactors to cause the formation of a shaped material suitable for specific clinical applications, such as a mandible shaped reactor for *in vitro* growth of a shaped bone graft substitute for the use in repair of fractured jaws. This invention further relates to a bone forming tissue that will remodel into load-bearing bone when implanted in the surgical repair of bone defects.

## Background of the Invention

Demineralized bone matrix (DBM) is widely used in the repair of pathologies associated with skeletal defects and periodontal diseases. This material is typically produced from cortical bone of long bones such as those bones found in the legs and arms of human cadaveric donors. The shafts of these long bones are cut using methods well-known in the field into small chunks (about 1-4 mm), and subsequently cleaned and ground into a finer bone powder. The resulting bone powder is typically in the particle range size of about 125 to 1000 microns and is demineralized by exposure to dilute (normally 0.4 to 0.6 N) hydrochloric acid or organic acids, calcium chelating agents, etc., as one skilled in the art would appreciate. This ground demineralized bone matrix material is known as demineralized freeze-dried bone allograft (DFDBA), demineralized bone allograft (DBA), demineralized bone matrix (DBM), and demineralized bone (DMB) and is currently being produced for use in orthopaedic, spinal fusion, and periodontal applications.

The use of DBM in the formation of new bone has been assessed using *in vivo* (usually a mouse or rat implant system), *in vitro* (cell culture or extraction and quantitation of bone forming molecules reportedly present in bone), and *in situ* (where the formation of new bone in patients has been assessed during clinical applications)

models. Methods of assessing this new bone formation and the effects of the demineralization process on new bone formation by DBM have been described. (Zhang et al., A quantitative assessment of osteoinductivity of human demineralized bone matrix, J. Periodontol. 1997;68:1076-1084 and Zhang et al., Effects of the demineralization process on the osteoinductivity of demineralized bone matrix, J. Periodontol. 1997; 68:1085-1092.) An *in vitro* assessment of the ability of DMB to induce cells towards an osteoblastic phenotype has also been described. (Wolfinbarger and Zheng, An *in vitro* bioassay to assess biological activity in demineralized bone, In Vitro Cell Bio. Anim. 1993;29A:914-916.)

DBM is assumed to primarily form new bone when implanted in animal models via an endochondral pathway. The implanted DBM is presumed to cause mesenchymal stem cells (typically undifferentiated fibroblasts) to migrate towards the implanted biomaterial(s). This induced chemotaxis results in cells infiltrating the implanted biomaterial(s) (DBM) where they are induced to undergo phenotypic changes from a fibroblastic cells phenotype to a chondrocyte phenotype and eventually to an osteoblast cell phenotype. These induced phenotypic changes have been reported to be due to the action of one or more small molecular weight proteins falling in the TGF-β family commonly called bone morphogenetic proteins (BMPs). As the change in cell phenotypes occurs, the cells synthesize and secrete collagens and other matrix forming proteins/glycoproteins laying down a cartilagenous matrix and finally an osteoid-like matrix which if left implanted in the animal long enough can be shown to mineralize-a process analogous to the formation of new bone.

If the implanted materials lack the cell inducing protein factors, only providing an environment suitable for cellular infiltration and cellular proliferation and differentiation, the implanted materials are deemed to be osteoconductive. If the implanted materials possess the cell inducing protein factors and provide an environment suitable for cellular infiltration and cellular proliferation and differentiation, the implanted materials are deemed to be osteoinductive. If the implanted materials already contain cells suitable for new bone formation, such as autogenously transplanted bone, the materials are deemed to be osteogenic.

## Summary of the Invention

The present invention is directed to a method of growing new bone or bone-like tissue under *in vitro* cell culture conditions comprising providing ground demineralized bone and bone-forming cells in a bioreactor under conditions sufficient to form bone or bone-like tissue suitable for transplantation by causing a flow of nutrient solutions into, through, and out of the bioreactor. The bone or bone-like tissue is formed by proliferation and/or differentiation of the bone-forming cells in the presence of the ground demineralized bone and under suitable bioreactor conditions.

The bone-forming cells are preferably selected from the group consisting of stem cells, fibroblast cells, periosteal cells, chondrocytes, osteocytes, pre-osteoblasts, and osteoblasts. The most preferred bone-forming cells are fibroblast cells and pre-osteoblasts. The bone-forming cells can be autograft, allograft or xenograft with respect to the intended recipient.

In accordance with the invention, the ground demineralized bone may be in the form of particles or fibers. The particles are about 50 microns to about 4 mm, preferably about 250 microns to about 710 microns. The fibers have a width of about 0.1 mm to about 0.5 mm, a thickness of about 0.05 mm to about 0.5 mm, and a length of about 1 mm to about 500 mm. If the ground demineralized bone is freeze-dried, it should be rehydrated. The invention provides that rehydration may occur either prior to or after being added in the bioreactor.

The invention further provides that additional components may be added to the bioreactor, such as collagen or hyaluronin, which may create a viscous bone-like matrix. Additionally, growth factors, such as vascular endothelial growth factor or differentiation factors such as bone morphogenetic proteins may be added.

The nutrient solution may comprise at least one of Dulbecco's modified Eagle's medium, fetal bovine serum, L-ascorbic acid, antibiotics, dexamethasone, beta-glycerolphosphate, glucose, glutamine, amino acid supplements, glutathione-ethyl ester, antioxidants, caspase inhibitors, and inorganic ions suitable for mineralization-related metabolic events.

The nutrients solution may be delivered to the ground demineralized bone and bone-forming cells by resorbable hollow fibers. The hollow fibers are also sufficient to remove metabolic waste products from the bioreactor.

In another aspect of the invention, nondemineralized bone may be added along with the demineralized ground bone. The ratio of demineralized ground bone to nondemineralized bone may be about 1:1 to about 20:1 or as necessary to control availability of biologically active agents and available volume for cell growth.

The present invention is further directed to the bone or bone-like tissue formed according the process disclosed herein. Moreover, implants comprising the bone or bone-like tissue is within the scope of the invention.

In yet another aspect of the invention, a device for the growth of new bone or bone-like tissue under *in vitro* cell culture conditions is provided. The device comprises a bioreactor, wherein the bioreactor comprises inlet and outlet ports for the flow of nutrient solutions, sample injection ports, optionally including hollow fibers for the delivery of nutrients and removal of wastes, wherein the bioreactor is capable of applying mechanical/electrical stimuli to the formed, or forming new bone.

Furthermore, a method for growing an extracellular matrix capable of forming bone when transplanted into a patient is described. The method comprises providing bone-forming cells in a bioreactor under conditions sufficient to promote the growth and differentiation of cells resulting in the formation of an extracellular matrix, wherein said conditions include the flow of nutrient solutions through the bioreactor. Preferably, ground demineralized bone is added to the bioreactor. The present invention further encompasses the extracellular matrix made by this process and a method of implanting bone into a patient in need thereof comprising transplanting the formed extracellular matrix into the patient under conditions sufficient to form bone.

## Brief Description of the Drawings

Figure 1 illustrates a bioreactor to be used in the *in vitro* growth of bone or materials that will turn into bone following transplantation into a recipient.

Figure 2 schematically depicts the bone plug formed *in vitro* in the bioreactor filled with ground demineralized bones and bone-forming cells.

Figure 3 describes the production of osteocalcin at different cell seeding densities over time indicating a significant osteocalcin increase after one week of incubation using the sample inner vessels consisting of dialysis membrane tubing. There was no significant difference of osteocalcin production for each dialysis tube with various cell seeding densities.

Figure 4 is a graph depicting alkaline phosphatase activity from the *in vitro* alkaline phosphatase assay as a function of various cell seeding densities of human periosteol (HPO) cells (million cells x $10^6$). These data demonstrate that HPO cells at a density of 1 x $10^7$ cells have significantly higher alkaline phosphatase activities than other groups with different cell seeding densities tested.

Figures 5A and 5B depict the cross-sections of bone-like tissue samples after three weeks in a bioreactor exhibiting large cuboidal-shaped cells with deposition of collagen and organic bone matrix.

**Description of the Preferred Embodiments**

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural references unless the context clearly dictates otherwise.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices, or constructs similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices, or constructs are now described.

The term "bioreactor" is intended to mean a contained or enclosed system or vessel for the culture of mammalian or vertebrate cells by which sterility, i.e., freedom from microbial contamination, can be achieved. Nutrient solutions can be aseptically delivered into the bioreactor and waste solutions can be aseptically removed from the bioreactor.

The term "newly formed bone" is intended to mean a matrix secreted by bone forming cells. This newly formed bone is best illustrated by histological evidence of

newly formed bone when demineralized bone is implanted intermuscularly in a nude mouse (or rat) bioassay system.

The term "osteoid" refers to that extracellular matrix capable of mineralizing to form load-bearing bone.

The term "bone tissue" is intended to include the organic phase or organic and inorganic phases of that tissue comprising a bone. Within the context of this invention, bone tissue can include newly formed bone, implant bone, and associated cells, bone marrow, bone marrow-like tissue, cartilage, and cartilage-like tissues.

The term "transplantable bone" is intended to include a nonmineralized, partially mineralized, or fully mineralized viable construct produced, using a bioreactor, that is nonload-bearing, partially load-bearing, or fully load-bearing at the time of transplantation.

The term "implantable bone" is intended to include a nonmineralized, partially mineralized, or fully mineralized nonviable or viable construct produced, using a bioreactor, that is nonload-bearing, partially load-bearing, or fully load-bearing at the time of implantation.

The terms "stress" and "strain" are intended to include forces applied to the cells and matrix contained in a bioreactor that contribute to manipulation of phenotype of the cells contained therein. As used in the present invention, strain is expected to be applied to the cells and matrix in the bioreactor through forces applied to and within the bioreactor.

The term "hollow fiber" is intended to include tubular structures containing pores of defined size, shape and density for use in delivering nutrients (in solution) to cells contained within a bioreactor and for removal of waste materials (in solution) from cells contained within a bioreactor. For purposes of the present invention, hollow fibers may be constructed of a resorbable or nonresorbable material.

The term "nutrient solution" is intended to include solutions entering a bioreactor and containing those nutrient materials essential to the culture of mammalian or vertebrate cells. Nutrient solutions may also contain additives that affect specific changes in phenotype of cells under culture or to contribute to changes in the matrix structure of the forming newly formed bone, for example, mineralization.

The term "waste solution" is intended to include solutions exiting a bioreactor and containing waste byproducts of cellular metabolism. The concentrations of waste byproducts, for example, ammonia, lactic acid, etc. and residual levels of nutrients such as glucose, in the waste solution can be used to assess the levels of metabolic activity of cells being cultured in a bioreactor.

The present invention provides a method of growing bone *in vitro* involving providing a biomaterial, such as ground demineralized bone, suitable for inducing cells to form an extracellular matrix and cells capable of forming bone or bone-like biomaterials, and placing the biomaterial and bone-forming cells in close association under conditions suitable for forming bone or bone-like biomaterial. In particular, the ground demineralized bone and bone-forming cells are preferably placed in a bioreactor capable of simulating the nutrient flow and waste removal present within an implant site. The flow of nutrient solutions into, through, and out of the bioreactor permit the associated ground demineralized bone and bone-forming cells to form into bone or bone-like biomaterial suitable for transplantation.

The biomaterial, ground demineralized bone, is capable of inducing selected cell types to form an extracellular matrix consistent with the osteoid materials comprising the organic phase of bone tissue when implanted in heterotopic or orthotopic sites in a living organism. Ground demineralized bone is obtained in manners known in the art and may be available in any form, including as particles or fibers. Ground demineralized freeze-dried bone particles may be used in any particle size suitable for inducing the growth of bone in a bioreactor, such as from about 50 microns to 4 mm, preferably, about 125 microns to 850 microns, and most preferably, about 250 microns to 710 microns. Ground demineralized bone fibers may be produced in known manners, such as by skiving or shaving the surface of the cortical bone to produce short fibers that easily entangle. The fibers are suitable for growing bone in a bioreactor and preferably have physical dimensions of about 0.1 mm to 0.5 mm in width, 0.05 mm to 0.5 mm in thickness, and 1 mm to 500 mm in length. The bone used to make the ground demineralized bone may be processed in known manners prior to forming the ground demineralized bone used in connection with the present invention. For example, the bone may be treated with enzymes to partially digest the organic components of the bone, such as collagenase,

papain, protease, hyaluronidase, endonuclease, lipase, and/or phosphatase, or organic acids, such as acetic or citric acid. Alternatively, the bone may be partially digesting by fragmenting the covalent bonds in the individual collagen molecules contained in the demineralized bone. The covalent bond breakage of the formed fragments of a collagen molecule is in the range of about 2 and about 50, sufficient to modify the resorption rate of the demineralized bone. Subsequent to forming the fibers or particles, the fibers and particles are demineralized by exposure to dilute (about 0.4 to 0.6 N) hydrochloric acid or organic acids, calcium chelating agents, etc., as one skilled in the art would appreciate. Alternatively, non-acid chelators of calcium, such as ethylene diamine tetraacetic acid (EDTA), may be used to demineralize the bone.

In addition, the weight percent residual calcium in ground demineralized bone is a factor in defining the bioavailability of bioactive molecules, for example, bone morphogenetic proteins (BMPs for example), to the cellular population contained within the bioreactor. A suitable amount of residual calcium is in the range of about 0-8 weight percent, preferably about 1-4 weight percent, and most preferably about 2 weight percent.

The ground demineralized freeze-dried bone particles are added aseptically to the bioreactor. They may be directly added to the bioreactor in a freeze-dried state and rehydrated in the bioreactor or rehydrated in culture medium prior to addition to the culture chamber of the bioreactor. The ground demineralized bone may be added alone or in combination with other components that do not inhibit the effect of the ground demineralized bone to induce bone formation. For example, ground nondemineralized bone may be added with ground demineralized bone. In such cases, the ground demineralized bone to nondemineralized bone is added in a ratio of about 1:1 to about 20:1, preferably about 8:1, and most preferably about 3:1. The ground nondemineralized bone may take any form, e.g., particles or fibers, and typically will have similar physical dimensions as the ground demineralized bone.

Particle size ranges used in the bioreactor determine the "void volume" or available volume outside of the ground demineralized bone particles in which the bone-forming cells and other components may be added. The spacing of the ground demineralized bone particles in the bioreactor effects the ability of the bone-forming cells to differentiate and/or proliferate based on its contact with bone-forming cells and

infiltration within the voids between the particles. In particular, bone particle spacing will differ depending on whether or not the bone particles are rehydrated prior to addition to the bioreactor growth chamber. For example, addition of the bone particles to the bioreactor growth chamber in a freeze-dried state is relatively easy and the particles will pack tightly filling the available space. Subsequent rehydration of these bone particles in the bioreactor will cause the bone particles to swell to a tighter state of packing due to rehydration than if added in the hydrated state. In that the bone-forming cells will need to be added to the rehydrated bone matrix void volume (that volume outside of the bone particles) after being rehydrated in the bioreactor, the tighter packing may hinder infiltration of the void volume present throughout the bioreactor. Rehydration of the freeze-dried bone particles prior to addition to the growth chamber of the bioreactor makes it more difficult to add the bone particles to the bioreactor and achieve a uniform packing geometry. However, this approach allows the bone-forming cells to be added to the rehydrated bone particle suspension and thus the bone particles and fully dispersed cells can be more uniformly distributed within the growth chamber and are less likely to contribute to damage to the hollow fibers present within the growth chamber. Centrifugal forces can be used to cause the rehydrated bone particles and cells to pack throughout the growth chamber with excess fluids removed from the packing port. In accordance with the present invention, the ground demineralized bone particles may be rehydrated in the bioreactor or prior to being added to the bioreactor. Preferably, the particles are rehydrated and mixed with cells prior to addition to the bioreactor.

The "bone-forming cells" of the present invention are those cells suitable for the induction of new bone formation when infiltrated with ground demineralized bone in a bioreactor and include those cell types suitable for differentiating into bone cells or suitable for forming a matrix similar to osteoid of forming new bone. Suitable cell types include, but are not limited to stem cells, fibroblast cells, periosteal cells, chondrocytes, osteocytes, pre-osteoblasts, and osteoblasts. Preferably, the stem cells are multipotent, the fibroblast cells are undifferentiated, the periosteal cells are partially differentiated, and the chondrocytes or osteocytes are differentiated. In the case of differentiating cell types, such as fibroblasts or stem cells, these cell types may be placed in close proximity to the ground demineralized bone, which, in the bioreactor and under appropriate

conditions, will cause the cells to differentiate into bone cells. In the case of cell types suitable for forming an osteoid-like matrix, such as osteoblasts or chondroblasts, such cell types may be placed in close proximity to the ground demineralized bone in the bioreactor and under appropriate conditions, will cause the cells to synthesize matrix similar to osteoid of forming new bone. The type of cells selected for *in vitro* bone growth is dependent upon the desired time frame for new bone formation, seeding cell densities, and nutrient medium provided. Use of a potential recipients own cells in the formation of the bone or bone-like biomaterial will result in a tissue unlikely to be rejected for some immunological reason, rendering the transplantable newly formed bone autogenous in nature. Use of allogenic cells in the formation of new bone with subsequent implantation can be achieved by decellularizing any newly formed bone or bone-like structure prior to implantation using any decellularizing technology known in the art depending on the desired characteristics of the acellular bone or bone-like structure desired for a given clinical application.

The bone-forming cells are added to the void volume space of the packed demineralized bone particles. The cell density of the bone-forming cells may be in the range of from about $10^2$-$10^8$ per ml , preferably $10^3$-$10^6$ per ml, and more preferably about $10^4$-$10^5$ per ml. The density of bone-forming cells added depends on several factors. For example, previous cell culture work in development and validation of *in vitro* bioassays for assessing the osteoinductive potential of demineralized bone demonstrated the importance of cell density difference depending on the phenotypic status of the cells. (Wolfinbarger, L and Y. Zheng. 1993. An *in vitro* bioassay to assess biological activity of demineralized bone. In Vitro Cell Dev. Biol. Anim. 29:914.) Less differentiated cells (e.g., dermal fibroblasts), where proliferation constituted a component of the differentiation process, involved a lower seeding density in *in vitro* bioassays than more differentiated (periosteum derived cells, for example) cells. Presumably, cells more differentiated along the pathway leading from a "stem-like" cell to a differentiated cell phenotype proliferated less well (longer population doubling times of approximately 40 hours) than less differentiated cells (shorter population doubling times of approximately 12 hours) and could be seeded at higher cell densities when used in an *in vitro* bioassay. Consequently, seeding densities of cells in the bioreactor depends in part on the

phenotype of the cells added to the bioreactor, the availability of biologically active materials, and the culture medium used. In addition, seeding cell density in the bioreactor depends on the ability to deliver nutrients to the cells and remove waste byproducts from the bioreactor culture chamber.

The bioreactor can be in virtually any shape based on the shape of the bioimplant desired as a newly formed bone or structure that will form load-bearing bone when implanted clinically. The wall of the bioreactor can be deformable and contained within a nondeformable chamber such that positive and negative pressure environments can be applied between the inner wall of the nondeformable chamber and the outer wall of the deformable chamber such that the volume of the bioreactor containing the demineralized bone, cells, and matrix can be decreased or increased over time to simulate stress and strain application to the bone matrix being formed.

The demineralized bone and bone-forming cells can be preloaded into the bioreactor in the presence, or lack thereof, of a viscous matrix designed to provide attachment sites for the cells and/or to restrict diffusion of synthesized osteoid forming molecules. The viscous nature of the matrix may be obtained by the incorporation of polymers, for example, collagenous, hyaluronin, or similar resorbable or nonresorbable polymers.

Nutrients are delivered to the ground demineralized bone and bone-forming cells in the bioreactor and may impact the growth and differentiation of cells contained in the bioreactor. The nutrient solutions are selected to provide sufficient nutrition to the bone-forming cells to maintain viability, growth, and/or differentiation in the bioreactor. Those skilled in the art are capable of selecting an appropriate nutrient solution for the present invention. For example, media such as Dulbecco's modified Eagle's medium may be used and may be further supplemented with other suitable nutrients. Other suitable nutrients include fetal bovine serum, L-ascorbic acid, antibiotics, cell modulators such as dexamethasone, beta-glycerolphosphate, glucose, glutamine, amino acid supplements, inhibitors (or activators) of apoptosis such as glutathione-ethyl ester, antioxidants, caspase inhibitors, and cations and anions, e.g., magnesium, manganese, calcium, phosphate, chloride, sodium, potassium, zinc, and sulfate ions, and nitrates and nitrites. The concentration of fetal calf serum must not inhibit induced cell

differentiations due to diffusible agents from the dimeralized bone. The remaining concentration of components in the nutrient solution should be sufficient to promote growth and/or differentiation in the bioreactor and maintain viability of the bone-forming cells and the resulting bone or bone tissue.

In accordance with the present invention, the nutrient solutions may be modified during different phases of the process. For example, during initial culture, seeded cell densities will be minimal, especially for fibroblast cell seeding cultures, and thus nutrient solutions may contain low concentrations of fetal calf serum to facilitate the role of growth and differentiation factors diffusing from the ground demineralized bone particles in modulating phenotypic changes in the added cells. Monitoring the concentration of the nutrients, such as glucose, glutamine, and amino acid supplements, via the eluent flow of medium allows for the determination of nutrient consumption permitting control of flow (delivery) of nutrients into the cell population. Moreover, waste products of metabolism, for example, ammonia and lactic acid, can be monitored via the eluent flow of medium from the bioreactor to determine the metabolic state/function of the resident cell population. Changes in cell phenotype during the culture phase can be monitored by sampling the eluent flow of medium from the bioreactor for proteins associated with specific cell phenotypes, for example, osteopontin and osteocalcin. Should it be desired, for example, to induce mineralization during a specific phase of the culture period, the medium can be amended with chemicals such as β-glycerolphosphate as a substrate for alkaline phosphatase and to serve as a source of phosphate to be complexed with calcium in the formation of crystalizable calcium salts such as hydroxyapatite. The levels of oxygen tension can be controlled by oxygenation of the nutrient medium being added to the cells being cultured in the bioreactor to manipulate the metabolic state of the cells during the culture phase such that mildly hypoxic conditions can be used to manipulate chondrogenesis and/or osteogenesis. Manipulation of the ionic composition of the medium can be used to control hydrolytic enzyme degradation of demineralized bone matrix, enzyme mediated cross-linking of the formed extracellular matrix being synthesized by the resident cell population, and the osmotic balance of the nutrient solution. Induction and/or inhibition of cellular apoptosis can be controlled by the addition of inhibitors (or activators) of apoptosis such as glutathione-ethyl ester,

antioxidants, and caspase inhibitors or activators. For example, use of allogenic cells may required induction of apoptosis to produce a cellular formed bone tissue.

The nutrients may be delivered in any manner suitable for the formation of bone in the bioreactor. For example, resorbable hollow fibers can be used to deliver nutrients and remove metabolic waste products during the cellular proliferations and/or differentiation process. The nutrient solutions used can be sequentially introduced into the bioreactor growth chamber as needed to induce cellular morphogenesis, growth, secretion of osteoid biomaterials, and/or to cause mineralization of the formed matrix as desired depending on the type of implantable bone material desired. The resorbable hollow-fibers used to deliver nutrients and remove wastes from the bone forming part of the bioreactor provide an opportunity to leave a series of hollow tube-like openings within the formed bone tissue through which the formed bone tissue can be vascularized. Growth factors such as vascular endothelial growth factor (VEGF) can be final delivered through these hollow fibers once the bone tissue has been formed to promote angiogenesis within the hollow structures following transplantation.

Delivery of nutrients and removal of waste products depends primarily on two factors: numbers of hollow fibers per unit volume of the culture chamber of the bioreactor and flow rates of nutrient solutions through the hollow fibers. Although the flow of nutrient solutions through the hollow fibers will generate some minimal turbulent flow of solutions through the bulk volume of the growth chamber of the bioreactor, the primary mechanism for nutrient dispersal through the growth chamber and to the cells in culture will be diffusion. Diffusion of nutrients from capillary beds in tissue typically limits the provision of nutrients (for example oxygen, glucose, etc.) to a few millimeters from an individual capillary. Thus, it should be expected that cells located more than a few millimeters from a hollow fiber will receive less nutrients and exist in a greater concentration of waste byproducts than cells close to a hollow fiber.

Shear stress to cells present in the bioreactor due to flow of nutrient solution will be minimal. Thus, optional addition of mechanical stress and strain to the forming bone matrix will occur primarily via manipulation of the inner vessel in the bioreactor used to contain the demineralized bone, cells, and extracellular formed/forming matrix. This component of the bioreactor includes the option of placing an inner vessel constructed of

a deformable material within an outer vessel to which cyclic positive and negative pressure can be applied via a port in the outer vessel wall. It is to be expected that such positive and negative pressures will be minimal and designed to gently compress and expand the forming extracellular matrix in order to provide cyclic mechanical stimulation to the cells contained within the inner vessel of the bioreactor.

In addition to the cyclic mechanical stimulation to cells contained within the inner vessel of the bioreactor, the inclusion of a series of micro-electrodes within the inner wall of the inner vessel in liquid contact with the forming, or formed, extracellular matrix will allow cyclic, low-level, electrical stimulation of cells and/or the creation of a small electrical gradient from one end to the other end, or side to side, of the bioreactor for use in electrical stimulation of cellular metabolism during induced new bone formation. This cyclic electrical stimulation can occur concurrent with, or not concurrent with, other mechanical or media changes to the forming, or formed, extracellular matrix containing the cells being manipulated to form new bone or bone-like tissue(s).

One aspect of the present invention is practiced by sterilizing all aspects of the bioreactor (tubing, fittings, valves, reagent (solution) containers, filters, sampling ports, bioreactor components, etc.). The bioreactor 120 as set forth in Figure 1 is aseptically assembled such that the hollow fibers 180 are connected to the inlet end-plate 170 and drawn through the tubular inner vessel 190 of the bioreactor 120 allowing the tubular inner vessel of the bioreactor to be attached to the inlet end-plate 170 forming a water-tight seal. The ground demineralized bone is added into the inner-most volume of the inner vessel 140 before or following rehydration until it fills the inner-most volume. If the ground demineralized bone is rehydrated prior to addition to the inner-most volume, it is mixed with the cells to be used at an appropriate seeding density, i.e. number of cells/unit volume of extra-particle space. If the ground demineralized bone is not rehydrated prior to addition to the inner-most volume, the bone will need to be rehydrated prior to addition of cells once the bioreactor is fully assembled. The non-connected end of the hollow-fibers is then carefully attached to the outlet end-plate 160 forming a water-tight seal. This inner vessel 190 is now ready for insertion into the outer vessel component of the bioreactor. This is accomplished, in one aspect of the present invention, by sliding the outer most diameter of one of the end-plates through the internal

lumen of the outer vessel until the remaining end-plate can form a water-tight seal with the inner diameter of the outer vessel. As an alternative method, the assembled inner vessel can simply be inserted into the outer vessel by guiding (pressing) the end-plates into the guide holes present in the inner faces of the outer vessel. Once the bioreactor is assembled, the ground demineralized bone can be rehydrated, if not already done so, and cells added via the injection ports 110. The bioreactor 120 is attached to the inlet and outlet tubing and the flow of nutrient solution from the nutrient reservoir 150 through the hollow fibers is initiated. The flow is initiated and maintained in manners known in the art, but is preferably conducted centrifugal forces or a pump 130 sufficient to cause the flow of media and waste products through the bioreactor 120. Initiation of flow of nutrient solutions is important in that the cells contained in the bioreactor are labile to nutrient deprivation and thus the time between addition of cells to the bioreactor and initiation of nutrient solution flow should not exceed a time in which the specific cell population in the nutrient solution used to pack them becomes depleted of nutrients or changes pH to an extend that the cells become metabolically stressed. Additional reagents may added through a reagent addition port 140 as described above. Moreover, the eluent flow 200 of medium from the bioreactor may be monitored to assess for proteins associated with bone formation, waste products, and nutritional capacity of the cells and demineralized bone, as described. The medium may also be recycled and recirculated into the nutrient reservoir 150 through a recycling inlet port 210.

The nutrient medium provided and the flow rate of this nutrient medium will vary depending on cell type added to the bioreactor, the packing density of the demineralized bone, presence/absence of a pre-added "extracellular matrix", and numbers and kinds of hollow fibers contained within the inner vessel of the bioreactor. Nutrient flow will continue until such time as it has been previously determined that the appropriate matrix (structure) has been obtained. At this time, the bioreactor is aseptically dismantled and the bone or bone-like structure aseptically removed for further use.

The formed new bone can consist of a nonmineralized and nonload-bearing osteoid-like material that will mineralize when transplanted into a heterotopic or orthotopic site in a patient or a partially mineralized and partially load-bearing osteoid material that will further mineralize when transplanted into a patient. Given time, it is

also possible to produce an almost completely mineralized bone-like tissue that will be load-bearing when implanted clinically.

In another aspect of the invention, the demineralized ground bone and bone-forming cells may form an extracellular matrix that is capable of forming bone when implanted in a patient. In this manner, the demineralized bone and cells may be gelled in a viscous material and have non-loading bearing implantable material that will form *in vivo* similar to the *in vitro* bone-forming process described above.

The bone, bone-like tissue, and extracellular matrix made according to the present invention is suitable for transplantation into a patient in need thereof. As one having ordinary skill in the art would appreciate, the bone, bone-like material or tissue, and extracellular matrix can be made into a desired shape that the body will remodel into the appropriate bone when implanted into a patient in some clinical application. As shown in Figure 2, a bone plug 210 is formed corresponding to the shape of the bioreactor 220 filled with demineralized bone cells and bone-forming cells in accordance with the present invention.

## EXAMPLES

The following examples are for purposes of illustration only and are not intended to limit the scope of the appended claims.

**Example 1**: Growth of new bone using a sample inner vessel consisting of dialysis membrane tubing in a circulating nutrient solution

The objective of this study is to investigate the new musculoskeletal tissue formation by using optimal amount of ground demineralized bone matrix (DBM) and optimal number of mammalian cells in a sample inner vessel consisting of dialysis membrane tubing.

Dialysis tubes (Spectra/Por DispoDialyzers: #135068) having a pore size of MWCO 25,000, 500ul, from Spectrum Laboratories, Inc. made with material, such as regenerated cellulose or cellulose ester, was used for musculoskeletal bone tissue regeneration. The hydrogen peroxide in sterile dialysis tubes were taken out and the tubes were submerged in tissue culture media (DMEM with 2% fetal bovine serum) for 1-2 hours in order to remove all remnants of hydrogen peroxide.

Human ground demineralized bone matrix (DBM) was prepared as known in the art and weighed aseptically.

Human skin fibroblasts (Hs27), CRL-1634-FL, American Type Culture Collection (ATCC), were obtained from a normal newborn black male and cryopreserved at ATCC. The cell line was revived and propagated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) incubated at 37°C in a $CO_2$ incubator with 5% $CO_2$. Cells at passage 18 to 24 were used for the following study.

Bone Growth Setup: 100 mg of ground DBM were weighed aseptically and rehydrated with various concentrations of Hs27 cell suspension: 0.5, 1.0, 2.0, or 5.0 million cells in 0.5 ml of DMEM containing 2% FBS. The DBM and cell mixtures were transferred into the prepared dialysis tubes. All tubes were submerged in DMEM containing 2% FBS, 50 μg/ml ascorbic acid, 1 μM dexamethasone, and 50 mM beta-glycerophosphate. The dialysis tubes were incubated in a media container, or in a media container which stays on stir plate to give constant mixing speed, or the dialysis tubes were incubated in a media flow chamber which controls the media flow rate for dialysis tubes by peristaltic pump. All cultures were kept at 37°C in a $CO_2$ incubator with 5% $CO_2$ for 7 weeks and culture media with supplements were changed once a week.

For each week, the culture media were taken out for osteocalcin quantification, the DBM and cell mixtures from dialysis tubes were taken out for histological analysis, alkaline phosphatase quantification, and genomic DNA quantification.

"Bone Plugs" with average sizes of 0.5x0.5x1.0-1.5 cm were generated after 3-4 weeks of incubation using the sample inner vessels consisting of dialysis membrane tubing. (Fig 1)

Osteocalcin Quantification: The culture media from container were taken out each week before media changing. Intact human osteocalcin ELISA kits from Biomedical Technologies Inc. were used to measure only intact osteocalcin, which was synthesized *de novo* by the osteoblasts. This assay is a sandwich ELISA, which utilizes biotinylated monoclonal antibodies, directed toward the amino- and carboxy- terminal regions of osteocalcin. It recognizes only intact osteocalcin, requiring the full 49 amino acid residue protein for detection. Then horseradish peroxidase conjugated streptavidin

attaches to the biotinylated antibody, which binds to the intact osteocalcin. The horseradish peroxidase can utilize the substrate tetramethylbenzidine (TMB) to generate product, which has the absorbance at 450 nm. The sample osteocalcin concentrations were calculated according to the standard curve generated for each assay.

Osteocalcin production increased significantly after 1 week of incubation using the sample inner vessels consisting of dialysis membrane tubing as shown in Figure 3. There was no significant difference of osteocalcin production for each dialysis tube with various cell-seeding densities.

Alkaline Phosphatase Assay: The dialysis tubes were taken out each week, and the DBM/cell mixtures were cut into half and weighed, then half tissue was transferred into ice-cold ultra-pure water and left on ice while sonicating at 9-10 watts for 30 seconds. Following a brief centrifugation (100g for 2 minutes), the aqueous phase was transferred to clean borosilicate glass tubes for bacinchoninic acid (BCA) protein assay and AP assay.

AP assay is a colorimetric method that utilizes para-nitrophenol phosphate as substrate to form p-nitrophenol. The rate of increase in absorbance at 405 nm due to the formation of p-nitrophenol is directly proportional to the alkaline phosphatase activity. Increased levels of AP activity for the DBM/cell mixture were the early marker of osteoblast differentiation.

Genomic DNA Quantification: Genomic DNA was extracted from DBM/cell mixtures by using Nucleon Extraction Kit from Amersham Life Science and quantified by PicoGreen dsDNA quantification kit (Molecular Probes, Inc) that utilizes an ultrasensitive fluorescent reagent, which is a nucleic acid stain for double-stranded DNA in solution. Samples were excited at 485 nm and the fluorescence emission intensity was measured at 538 nm using a spectrofluorometer. Eukaryotic cell genomic DNA standard was generated by plot fluorescence emission intensity versus DNA concentration. The sample genomic DNA concentrations were calculated according to the standard curve.

Histological Analysis: The DBM/cell mixtures were put into 10% formalin immediately after dialysis tubes were taken out of the container. Following 48 hours fixation, the tissues were embedded in paraffin and sectioned. The sections were stained

by Hemotoxilin and Eosin method. Calcium depositions were detected by Alizarin Red staining. Mason's Trichrome method was used for collagen presence demonstration.

H&E staining revealed the changes in Hs27 fibroblast morphology and new extra-cellular matrix (ECM) production that appeared to be "osteoid" formation. Masson's Trichrome staining suggested that the new ECM contained significant quantities of collagen. Alizarin Red staining revealed calcium deposition in newly formed ECM.

**Example 2**: Growth of new bone using a prototypic hollow-fiber containing bioreactor.

This experiment was conducted to determine the optimal ratio of seeding density of human periosteal (HPO) cells to demineralized bone matrix (DBM) for maximum new bone growth in hollow-fiber containing bioreactor.

The bioreactor was constructed from glass tubing (inner diameter, 5 mm; length, 50 mm) and contained forty porous regenerated cellulose hollow fibers (outer diameter, 216 μm; inner diameter, 200 μm; MWCO of 18,000; Spectra/Por® Cat#132226 from Spectrum Laboratories, Inc.; Laguna Hill, CA). The hollow fibers were embedded in biomedical grade silicon rubber (Nusil Silicone Technology, Carpenteria, CA). All the material was sterilized with ethylene oxide before usage.

Human ground demineralized bone matrix (DBM) was prepared in accordance with known methods and weighed aseptically.

Periosteum from a 55 years old female femur was acquired during routine tissue procurement by LifeNet staff with consent of the next kin. The periosteum was washed 3 times with Minimum Essential Medium Eagle (MEM, Sigma) containing 200 units/ml penicillin and 100 μg/ml streptomycin, cut into 1.0 mm x 1.0 mm pieces, and placed in T-25 culture flasks with the internal stratum osteogenicum layer facing toward the surface of the flasks. The preparation was cultured in alpha-MEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/ml)/streptomycin (50 μg/ml) in a 5% $CO_2$ incubator at 37°C. The outgrowing cells were transferred into T-75 flasks by detachment with a 0.025% trypsin and 0.05% EDTA solution and cultured in the same media and incubator. When cells reached confluence, they were split into new T-75 flasks at ratio of 1:4. Cells at passage 3 to 5 were used for the following study.

Hollow-fiber Bioreactor Setup: The bioreactor was constructed from chromatography column (inner diameter, 5 mm; length, 50 mm) and contained forty porous regenerated cellulose hollow fibers (outer diameter, 216 μm; inner diameter, 200 μm; MWCO of 18,000). The hollow fibers were embedded in biomedical grade silicon rubber. This hollow-fiber system was connected to a peristaltic pump with PharMed® tubing. The whole system was perfused with 70% isopropyl alcohol for 3 hours and perfused with saline for 3 hours before using for the following study.

Bone Growth Setup: To determine the optimal cell seeding density in the bioreactor system, human periosteal (HPO) cells were inoculated into the bioreactor at various cell density of $0.5 \times 10^6$, $1 \times 10^6$, $5 \times 10^6$, and $1 \times 10^7$ cells with DBM (1.5 cc or 500 mg). The culture medium used comprises Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml)/streptomycin (50 μg), 50 μg/ml ascorbic acid, 50 mM beta-glycerophosphate, 1 μM dexamethasone, and 2% fetal bovine serum (FBS). Two hundred and fifty ml of cell culture medium was recirculated with a medium flow rate of approximately 5 ml/min. After inoculation, the bioreactors were perfused using a peristaltic pump and maintained in a 5% $CO_2$/95% air incubator. After 5 days, the samples of cells with DBM were removed and *in vitro* alkaline phosphatase assay was performed. Figure 4 represents the various cell seeding densities of HPO cells and the activities of alkaline phosphatase from the *in vitro* alkaline phosphatase assay. These data demonstrate that HPO cells at a density of $1 \times 10^7$ cells have significantly higher alkaline phosphatase activities than other groups with different cell seeding densities tested.

To study the growth of new bone or bone-like tissue using hollow-fiber bioreactor system, the bioreactor was inoculated with $1x\ 10^7$ cells and DBM (1.5 cc or 500 mg) through either end into the extracapillary space of the bioreactor. Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml)/streptomycin (50 μM/ml), 50 μg/ml ascorbic acid, 50 mM beta-glycerophosphate, 1 μM dexamethasone, and 2% fetal bovine serum (FBS) was used as culture medium throughout the experiments. Culture medium was changed weekly. Two hundred and fifty ml of cell culture medium was recirculated with a medium flow rate of approximately 5 ml/min. Diffusive nutrient supply and removal of metabolic waste products across the membrane

of hollow fiber was advanced by constantly recirculating culture medium through the system using a peristaltic pump maintained in a 5% $CO_2$ incubator. After 3 weeks, samples were taken from the bioreactors, fixed in neutral buffered formalin, embedded in paraffin and sectioned. Sections were stained with Haematoxylin & Eosin. The results were illustrated in Figures 5A and 5B showing large cuboidal-shaped cells with deposition of collagen and organic bone matrix.

Alkaline Phosphatase Assay: The samples from 5 day incubation in bioreactor were transferred into ice-cold ultra-pure water and left on ice while sonicating at 9-10 watts for 30 seconds. Following a brief centrifugation (100g for 2 minutes), the aqueous phase was transferred to clean borosilicate glass tubes for bacinchoninic acid (BCA) protein assay and AP assay.

**Example 3:** Growth of new bone using a sample inner vessel consisting of dialysis membrane tubing in a circulating solution of nutrient solution.

Dialysis tubes (Spectrum, Spectra/Por) made with different membrane pore sizes (MWCO 10,000 – 25,000) and different material (regenerated cellulose or cellulose ester) are used for musculoskeletal bone tissue regeneration. The hydrogen peroxide in sterile dialysis tubes is removed and the tubes are soaked in tissue culture media, such as DMEM with 2% fetal bovine serum, for 1-2 hours in order to remove all remnants of hydrogen peroxide. Demineralized bone matrices are weighed aseptically and hydrated with cell suspension (dermal fibroblasts or periosteal cells) in RPMI 1640 tissue culture medium. The DBM and cell mixtures are introduced into dialysis tubes and the tubes are incubated in culture media containing 2% FBS, 50 μg/ml L-ascorbic acid, 1μM dexamethasone, and 50 mM beta-glycerolphosphate. The dialysis system is incubated either under static (i.e., the dialysis tubes are incubated in a media container), stirred dynamic (i.e., the dialysis tubes are incubated in a media container which stays on stir plate to give constant mixing speed), or fluid-flow dynamic (i.e., the dialysis tubes are incubated in a media flow chamber which controls the media flow rate for dialysis tubes by peristaltic pump) conditions.

During various time of incubation (1-7 weeks), the culture media is taken out from the containers for osteocalcin quantitation by ELISA, the tissues from the dialysis

tubes are taken out for histology analysis, alkaline phosphatase quantitation, percentage of calcium quantitation, and double strand DNA quantitation. The culture media is replaced by fresh media once a week to keep sufficient nutrients for cell growth and differentiation.

Each of the patents and publications cited herein are incorporated by reference herein in their entirety.

It will be apparent to one skilled in the art that various modifications can be made to the invention without departing from the spirit or scope of the appended claims.

10

What is claimed is:

1. A method of growing new bone or bone-like tissue under *in vitro* cell culture conditions comprising providing ground demineralized bone and bone-forming cells in a bioreactor under conditions sufficient to form bone or bone-like biomaterial suitable for transplantation by causing a flow of nutrient solutions into, through, and out of the bioreactor.
2. The method of claim 1, wherein the bone-forming cells is selected from the group consisting of stem cells, fibroblast cells, periosteal cells, chondrocytes, osteocytes, pre-osteoblasts, and osteoblasts.
3. The method of claim 2, wherein the bone-forming cells are fibroblasts or pre-osteoblasts.
4. The method of claim 1, wherein the ground demineralized bone is in the form of particles.
5. The method of claim 1, wherein the ground demineralized bone is in the form of fibers.
6. The method of claim 4, wherein the ground demineralized bone is freeze-dried.
7. The method of claim 6, wherein the ground demineralized bone is rehydrated prior to being provided in the bioreactor.
8. The method of claim 6, wherein the ground demineralized bone is rehydrated after being provided in the bioreactor.
9. The method of claim 1, wherein the bone-forming cells differentiate into bone or bone-like matrix.
10. The method of claim 1, wherein the bone-forming cells proliferate into bone or bone-like matrix.
11. The method of claim 1, further comprising adding collagen or hyaluronin to the associated mixture of ground demineralized bone and bone-forming cells.
12. The method of claim 11, wherein the bone-like matrix is viscous.
13. The method of claim 1, wherein the nutrient solution comprises at least one of Dulbecco's modified Eagle's medium, fetal bovine serum, L-ascorbic acid, antibiotics, dexamethasone, beta-glycerolphosphate, glucose, glutamine, amino

acid supplements, glutathione-ethyl ester, antioxidants, caspase inhibitors, cations, and anions.

14. The method of claim 1, wherein the nutrients solutions are delivered to the ground demineralized ground bone and bone-forming associated mixture by resorbable hollow fibers.

15. The method of claim 1, wherein the metabolic waste products are removed from the bioreactor by resorbable hollow fibers.

16. The method of claim 1, wherein growth factors are delivered to the formed bone or bone-like matrix.

17. The method of claim 16, wherein the growth factor is vascular endothelial growth factor.

18. The method of claim 2, wherein the ground demineralized bone particle size range is about 50 microns to about 4 mm.

19. The method of claim 18, wherein the ground demineralized bone particle size is about 250 microns to about 710 microns.

20. The method of claim 3, wherein the demineralized ground bone fibers have a width of about 0.1 mm to about 0.5 mm, a thickness of about 0.05 mm to about 0.5 mm, and a length of about 1 mm to about 500 mm.

21. The method of claim 1, wherein nondemineralized bone is provided with the demineralized ground bone.

22. The method of claim 8, wherein the ratio of mass of demineralized ground bone to nondemineralized bone is about 1:1 to about 20:1.

23. The method of claim 1, wherein the bone-forming cells are autograph, allograft or xenograft with respect to the intended recipient.

24. The method of claim 1, wherein the bone-forming cells are added to the rehydrated ground mineralized bone after the ground mineralized bone is added to the bioreactor.

25. The method of claim 1, wherein the bone-forming cells are added to the bioreactor prior to the addition of the ground mineralized bone to the bioreactor.

26. The bone or bone-like tissue formed according to the method of claim 1.

27. The bone or bone-like tissue formed according to the method of claim 2.

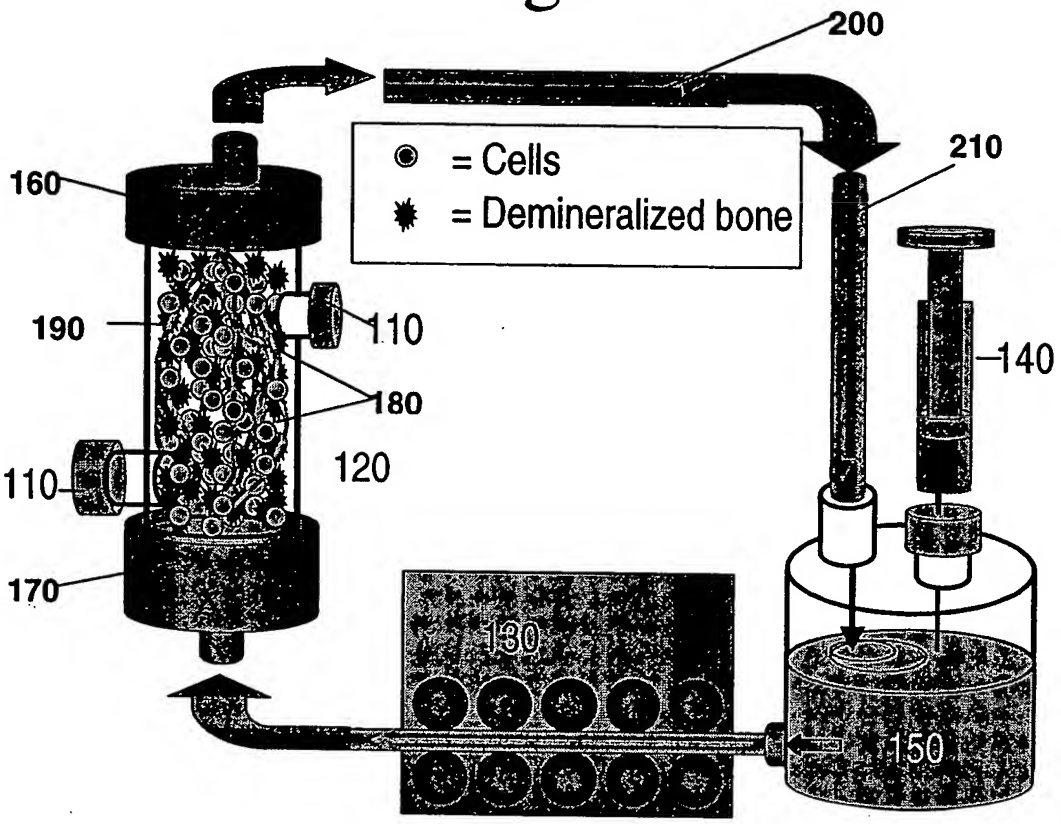
28. The bone or bone-like biomaterials formed according to the method of claim 12.
29. An implant comprising the bone or bone-like tissue of claim 26.
30. An implant comprising the bone or bone-like tissue of claim 27.
31. A device for the growth of new bone or bone-like tissue under *in vitro* cell culture conditions comprising a bioreactor, wherein the bioreactor comprises inlet and outlet ports for the flow of nutrient solutions, sample injection ports, optionally including hollow fibers for the delivery of nutrients and removal of wastes, wherein the bioreactor is capable of applying mechanical/electrical stimuli to the formed, or forming new bone.
32. A method for growing an extracellular matrix capable of forming bone when transplanted into a patient comprising providing bone-forming cells in a bioreactor under conditions sufficient to promote the growth and differentiation of cells into an extracellular matrix, wherein said conditions include the flow of nutrient solutions through the bioreactor.
33. The method of claim 32, further comprising adding ground demineralized bone to the bioreactor.
34. The method of claim 32, wherein the bone-forming cells is selected from the group consisting of stem cells, fibroblast cells, periosteal cells, chondrocytes, osteocytes, pre-osteoblasts, and osteoblasts.
35. The method of claim 34, wherein the bone-forming cells are fibroblasts and pre-osteoblasts.
36. The method of claim 33, wherein the ground demineralized bone is in the form of particles.
37. The method of claim 33, wherein the ground demineralized bone is in the form of fibers.
38. The extracellular matrix made by the method of claim 32.
39. The extracellular matrix made by the method of claim 33.
40. A method of implanting bone into a patient in need thereof comprising transplanting the extracellular matrix of claim 38 into the patient under conditions sufficient to form bone.

41. A method of implanting bone into a patient in need thereof comprising transplanting the extracellular matrix of claim 39 into the patient under conditions sufficient to form bone.

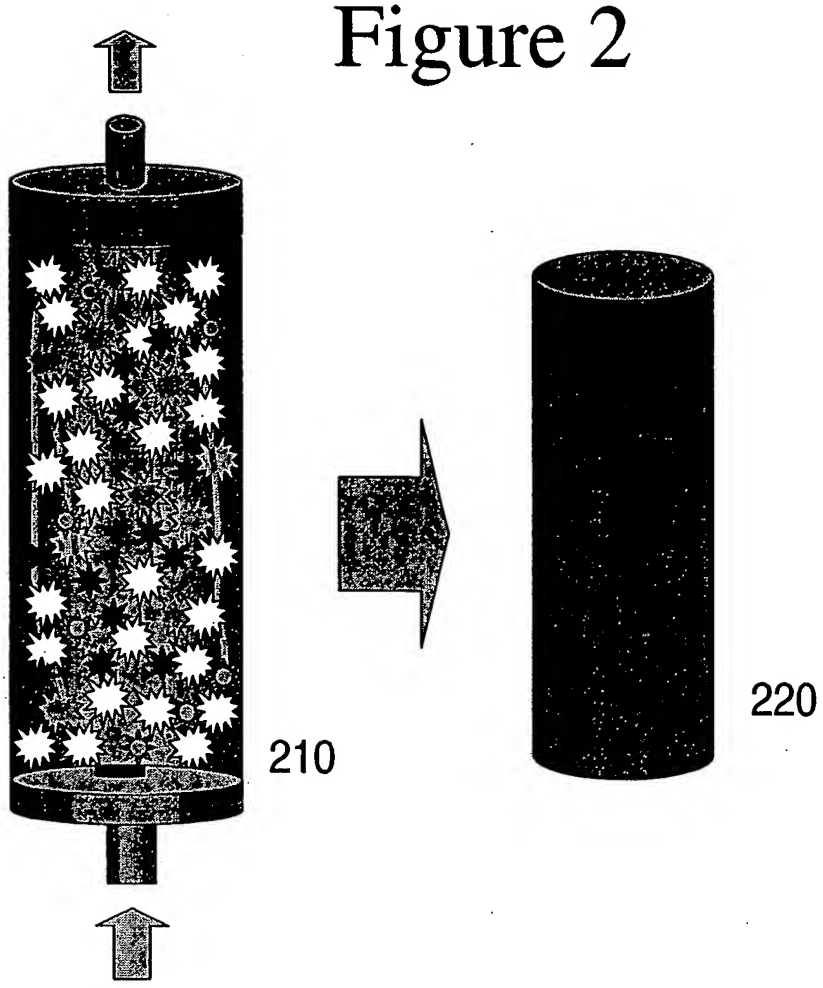
## ABSTRACT

This invention relates to the formation of a tissue-engineered material using *in vitro* cell culture, in a bioreactor system, in the presence of ground demineralized bone suitable for induction of new bone formation of suitable cell types. This invention further relates to the use of specific forms of bioreactors to cause the formation of a shaped material suitable for specific clinical applications, such as a mandible shaped reactor, for *in vitro* growth of a shaped bone graft substitute for the use in repair of fractured jaws. The bone and bone-like materials produced in according to the present invention are further described. Bone-like tissues made according to the invention are capable of remodeling into load-bearing bone when implanted in the surgical repair of bone defects.

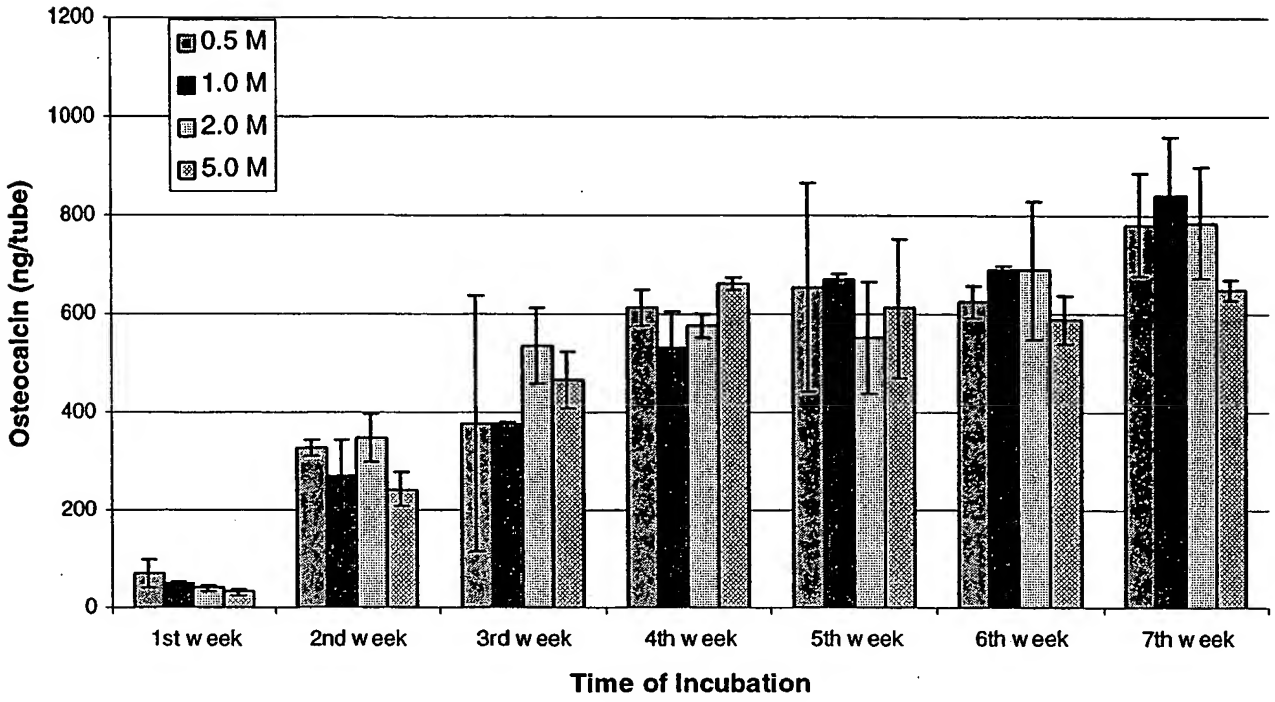
# Figure 1


200
210
= Cells
= Demineralized bone
160
190
110
180
120
110
140
170
130
150

# Figure 2



# Figure 3


0.5 M
1.0 M
2.0 M
5.0 M
Osteocalcin (ng/tube)
1200
1000
800
600
400
200
0
1st week
2nd week
3rd week
4th week
5th week
6th week
7th week
Time of Incubation

# Figure 4

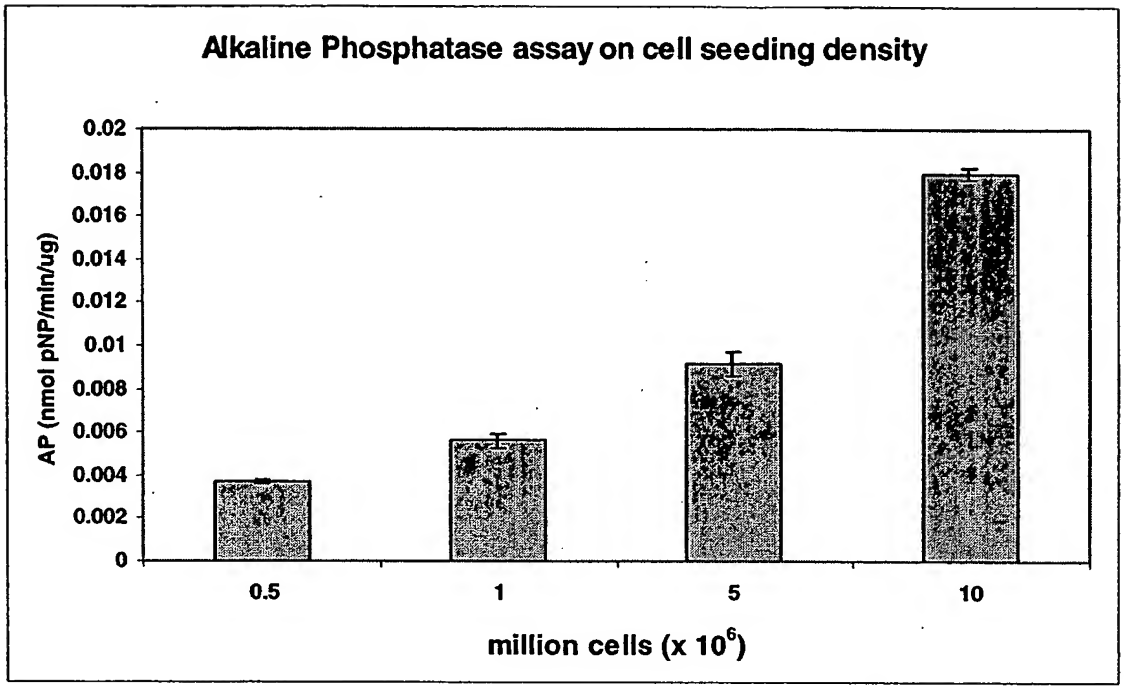

Alkaline Phosphatase assay on cell seeding density
AP (nmol pNP/min/ug)
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0.5
1
5
10
million cells (x 10^6)

**Figure 5A**

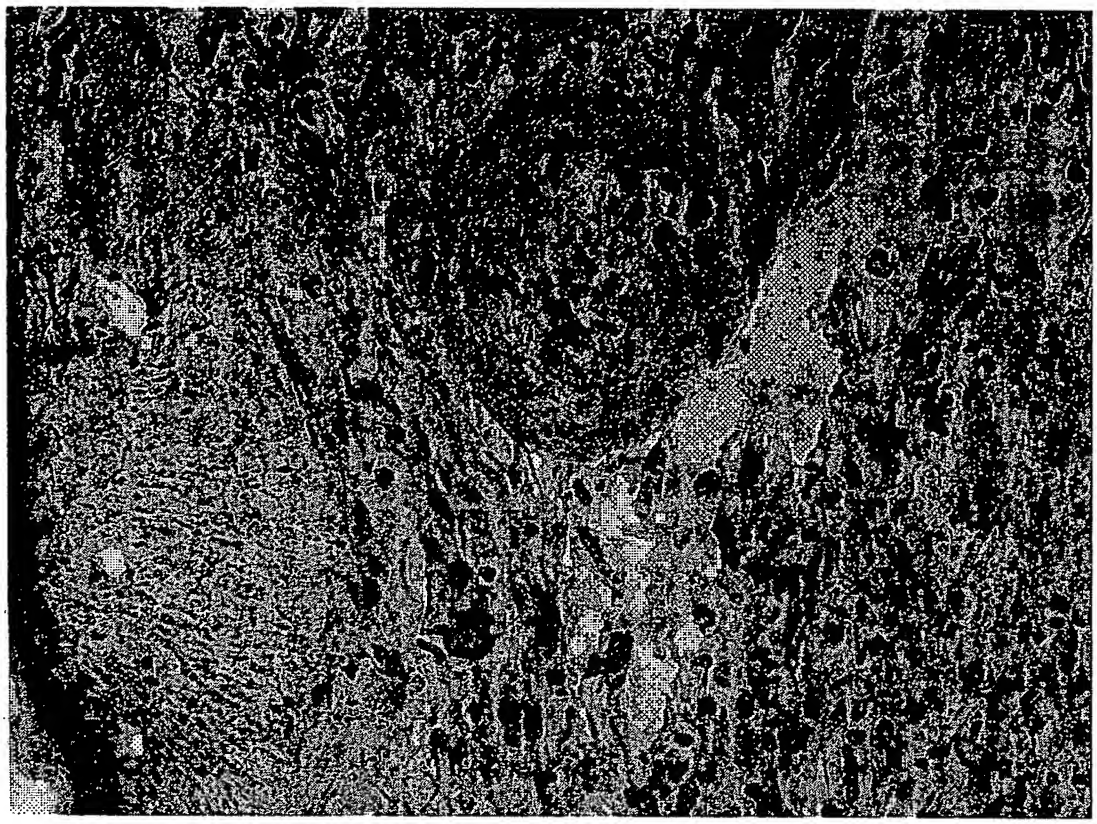


**Figure 5B**